Induced pluripotent stem cell research for Vanishing White Matter

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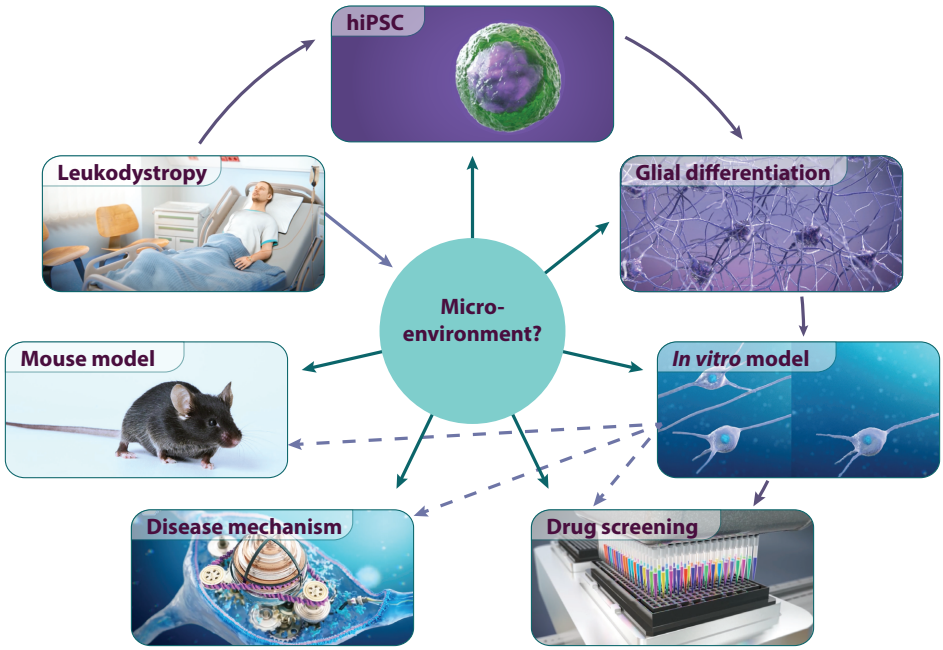
hiPSC
Leukodystropy
Glial differentiation
Micro-
environment?
Mouse model
In vitro model
Disease mechanism
Drug screening

Chapter 2

The healthy and diseased microenvironment regulate oligodendrocyte properties: implications for regenerative medicine

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Abstract

White matter disorders are characterized by deficient myelin or myelin loss, lead to a range of neurological dysfunctions, and can result in early death. Oligodendrocytes, which are responsible for white matter formation, are the first targets for treatment. However, many studies indicate that failure of white matter repair goes beyond the intrinsic incapacity of oligodendrocytes to (re)generate myelin, and that failed interactions with neighboring cells or factors in the diseased microenvironment can underlie white matter defects. Moreover, most of the WMDs show specific white matter pathology caused by different disease mechanisms. Here we review the factors within the cellular and the extracellular microenvironment regulating OL properties, and discuss stem cell tools to identify microenvironmental factors of importance for the development of improved regenerative medicine for patients with WMDs.

1. Introduction

The pathology of white matter disorders (WMDs) is characterized by deficient white matter (WM) or myelin loss. WMDs have different causes, such as injury, inflammation or genetic mutations. Currently there is urgent need for better treatment for patients with WMDs [1]. Cell replacement therapy is seen as one of the promising new treatment options. Since oligodendrocytes (OLs) are responsible for the generation of myelin in the brain, many studies focus on the development of OL replacement therapies [2,3]. Transplantation of glial progenitor cells in rodent models of myelin disease resulted in improved pathology and clinical phenotype [4-6]. However, while transplanted cells successfully spread throughout the brain, recovery is often insufficient. And in cases of successful transplantation, recovery was also ascribed to factors secreted by the transplanted cells, rather than cell replacement [7,8]. Moreover, brain pathology of WMD patients shows involvement of different neural cell types. Therefore, therapeutic strategies to regenerate white matter (WM) should comprise more than OL replacement [9].

WM structure and function is determined by a constant interplay between the OL intracellular properties and the local signals in the brain microenvironment. As a result, WMDs can be caused by an affected microenvironment, even though the OLs are intrinsically normal. On the other hand, malfunctioning OLs can lead to a changed microenvironment, which could cause WMD. In other words, as defected OLs disrupt normal interactions with their local milieu, the brain microenvironment will get into a diseased state over time as well. In this way, WMD pathology becomes multicellular. Hence, microenvironmental factors are important in pathology of all WMDs. Thus, before we can start designing new therapeutic strategies, we do not only need insight into basic disease mechanisms underlying WMDs, we also need understanding of how the microenvironment is primary and secondary involved. While some of these aversive microenvironmental factors easily reverse, others might need active modulation and should be resolved for successful treatment.

Here we give an overview of important components in the microenvironment that are known to regulate OL development and function. Furthermore, to identify new microenvironmental factors that influence OL properties and that are of importance in WM defects of particular WMDs, we discuss the use of new stem cell tools.

2. WMD cell therapy candidates

Cell replacement therapies have prospects for many WMDs, especially for the genetic WMDs as the OLs (and other neural cell types) carry genetic mutations. Before we describe microenvironmental components of importance to WM health, we first briefly discuss examples of genetic WMDs that are early candidates of future cell-based therapies.

2.1. Pelizaeus-merzbacher disease

Pelizaeus-merzbacher disease (PMD, OMIM: 312080) is characterized by diffuse hypomyelination or "tigroid" pattern of dysmyelination, and is caused by genetic changes in the X-linked proteolipid protein 1 (*PLP1*) gene, which is involved in the adherence and stabilization of compact myelin [10]. Most patients show duplications of the *PLP1* gene, which results in accumulation of PLP1 proteins in the lysosome affecting the viability of the OLs [11]. Missense mutations in *PLP1* cause a more severe type of PMD, where PLP proteins accumulate in the endoplasmatic reticulum, leading to reduced myelin formation and increased apoptosis of OLs [12]. In a first clinical trial, transplantation of human central nervous system (CNS) stem cells in PMD patients showed no adverse effects, which gives prospects for following clinical studies [13].

2.3. Alexander disease

Many genetic WMDs are caused by mutations in genes that are non-OL-lineage-specific, including Alexander disease (OMIM: 203450), which is caused by mutations in *GFAP*, a gene mainly expressed in astrocytes [14]. These mutations result in Rosenthal fibers, which are accumulations of clustered intermediate filaments in astrocytes [15]. Astrocyte dysfunction eventually leads to characteristic symmetric white matter abnormalities with frontal lobe predominance. Since Alexander disease is a typical WMD with cell-specific defects, cell replacement might be an attractive therapeutic strategy. However, the exact disease mechanism remains unknown. Dysfunction in glutamate uptake and potassium buffering, as well as production of inflammatory molecules and the activation of microglia, are suggested [16].

2.4. Vanishing white matter disease

Vanishing white matter disease (VWM, OMIM: 603896) is a progressive WMD, and results from mutations in the *EIF2B1-5* genes, which play a role in translation initiation and protein regulation [17]. Patients show diffuse myelin defects leading to cystic degeneration. While both oligodendrocytes and astrocytes look abnormal, there is increasing evidence suggesting that astrocytes are the primary affected cell type, eventually resulting in myelin loss [18,19]. VWM is suggested as early candidate for cell replacement therapy [1]. However, like other WMDs, VWM pathology is complex and involves changes in the microenvironment [19]. To design cell therapies, we need better understanding of how the microenvironment affects OL development and myelin formation.

3. Microenvironment regulates oligodendrocyte identity

During OL development, the processes of differentiation, migration and myelination comprise specific programs of gene expression, which have been studied and reviewed extensively [20,21]. These intracellular OL programs are influenced by many factors that come from outside the OL. For instance, generation of myelin sheaths is not only dependent on the developmental state of the OL, but also on the maturational state of the axon [22]. In addition, OLs show different myelination properties, depending on the location in the postnatal cortex [23]. OLs in the deeper cortical layers are more mature and produce more myelin than the OLs in the superficial cortical areas [23]. Similarly, WM regions stimulate maturation of OLs more than grey matter regions, regardless of the regional origin of the OLs [24]. Thus, different factors in the local microenvironment guide OL myelination.

Then again, different studies indicate that OLs have different intrinsic properties. Dorsally- and ventrally-derived OLs have different migration and settling patterns [25], and cortical- and spinal cord-derived OLs produce different myelin sheet lengths [26]. Furthermore, when transplanted into the grey matter, WM-derived OLs have higher maturation efficiency than grey matter-derived OLs [24]. This suggests that the early microenvironment to which the OL progenitors has been exposed to, also influences OL properties in adulthood. Indeed, OL progenitor populations emerge from different regions and at different time points in development [27]. During CSN patterning, different morphogens are secreted in a regionally- and temporally-restricted manner, resulting in specific anterior-posterior and rostral-caudal gradients. These gradients induce expression of particular transcription factors creating unique progenitor domains, where specific populations of progenitor cells emerge, including OL progenitor populations [27]. In summary, many OL populations exist in the brain, each with their own intrinsic capabilities, which are regulated by microenvironmental factors during early and late development.

To understand disease mechanisms underlying WMDs and to facilitate the development of regenerative medicine, we need better insight into mechanisms involved in OL development and WM formation. The microenvironmental factors regulating WM health, i.e. OL functioning, involve connections with local cells, the extracellular matrix (ECM), and cellular- and blood-derived factors. The cellular microenvironment involves neural cell types, including cell types of neuronal, astrocytic, and microglial cell lineages. The ECM surrounds the developing and mature OLs. The cellular and extracellular microenvironments, provide physical organization and signaling molecules, such as growth factors and neurotrophins. All these factors together are crucial for proper OL development, and determine OL properties in adulthood. In WMDs, these microenvironmental factors might be affected, which lead to affected OL function and subsequently failed WM (re)generation.

4. Cellular microenvironment

4.1. Neurons

Since the main function of OLs is myelinating axons, which is necessary for the efficient and rapid transmission of action potentials, the influence of neurons on OL development has been explored extensively [28]. Many studies showed that the number of neurons (i.e. axons) directly influences OL proliferation, survival and differentiation [28-30]. Next to cellular contact, neuronal activity can modulate the number of OL progenitors and the amount of myelin formed [31,32]. Change in the activity of the neurons affects the release of neurotransmitters. When unmyelinated axons fire action potentials, neurons can release the neurotransmitter glutamate, which upon binding of glutamate receptors on OLs can induce the myelination processes [33]. However, glutamate receptor properties depend on the developmental stage of the OL, and on the location in the brain [34]. It has been shown that prolonged glutamate receptor activation on OL progenitors causes calcium overload, and eventually cell death, while mature OLs stay unaffected [35]. Therefore, since different glutamate receptors have specific regulating roles, glutamate is suggested to have dynamic functions during OL development [36]. Next to glutamate, also other neurotransmitters affect OL progenitor development **(table 1)**. For example, several studies demonstrated regulating actions of GABA during OL development [37]. It has been shown that GABA signaling can modulate synaptic communication with hippocampal interneurons [38] and OL progenitor proliferation and migration *in vitro* [39]. Apart from cellular contact and neuronal activity, neurons also secrete many factors that regulate OL development via direct and / or indirect signaling **(table 2)**.

Name	Binding partner	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
glutamate	GluR, AMPAR, KAR, NMDAR, GLT-1, GLAST		+ / -	+	+	-	+
GABA	$GABA_AR$, $GABA_BR$		+ / -	+ / -	+		+
serotonin	5-HT2AR				-	-	-
adenosine	$A_{1A}R$, $A_{2A}R$			+			+
Acetyl-choline	nAChR, mAChR		+[40]	+	+ / -	+	
dopamine	DRD2, DRD3	-	+			+	
LINGO-1	NgR, p75				-		-
Nogo-A	NgR				+		-
Sema-phorins (SEMA)	plexins			+ / -	+ / -		+ / -
netrin-1	DCC, UNC5H1			-	+		+ / -
ephrins	EphA, EphB receptors			+			
thyroxine (T4), triiodo-thyronine (T3)	NR1A1, NR1A2				+		+
iron (Fe)	transferrin, ferritin	+	+		+	+	+
interleukins (IL)	IL receptors	+	+ / -		+	+ / -	+
leukemia inhibitory factor (LIF)	LIFR	+	+		+	+	+
tumor necrosis factor α (TNF-α)	TNFR1, TNFR2		+		-	-	+ / -
interferon-gamma (IFN-γ)	IFNGR1, IFNGR2		+ / -		-	+ / -	-

Table 1. **An overview of the microenvironmental factors predominantly derived from neurons or blood, that have been reported to regulate OL progenitor characteristics.** This overview is based on 100 experimental studies, which showed regulating effects of the presented neuron- or blood-derived factors on processes of OL development. This is not a complete list; many other studies have been performed. +, Positive regulation; −, negative regulation; A1AR, adenosine receptor 1A; A2AR, adenosine receptor 2A; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor; DCC, deleted in colorectal cancer; DRD, dopamine receptor D; Eph, erythropoietin-producing human hepatocellular; GABAAR, GABAA receptor; GABABR, GABAB receptor; GLAST, glutamate aspartate transporter; GLT, glutamate transporter; GluR, glutamate receptor; 5-HT2AR, 5-hydroxytryptamine receptor 2A; IFN-γ, interferon-γ; IFNGR, IFN-γ receptor; KAR, kainic acid receptor; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LINGO, leucine rich repeat and immunoglobin-like domain-containing protein; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NgR, Nogo receptor; OL, oligodendrocyte; NMDAR, N-methyl-d-aspartate receptor; NR1A1, nuclear receptor subfamily 1, group A, member 1; NR1A2, nuclear receptor subfamily 1, group A, member 2; TNF-α, tumor necrosis factor-α; TNFR, TNF-α receptor; UNC5H1, uncoordinated-5 homology 1.

Name	Receptor	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
platelet-derived growth factor AA (PDGF-AA)	PDGFαR	+	+	+	-		+
insulin-like growth factor 1 (IGF-1)	IGF1R	+	+		+	+	+
fibroblast growth factor 2 (FGF2)	FGFR1-4	+	+		+ / -		+ / -
epidermal growth factor (EGF)	EGFR	+	+	+	+ / -	+	+
ciliary neuro-trophic factor (CNTF)	CNTFαR		+		+	+	+ / -
brain-derived neuro-trophic factor (BDNF)	TrkB, P75		+ / -		+		+
glia derived neuro-trophic factor (GDNF)	GFRα1	+	+		+		+
nerve growth factor (NGF)	TrkA, p75		+	+		+ / -	-
Neuro-trophic factor 3 (NT3)	TrkB, TrkC		+		+	+	+ / -
neuregulin (NRG)	ErbB	+ / -	+	+	+ / -	+	+
SDF-1	CXCR4			+	+		+

Table 2. **An overview of the glycoprotein growth factors and neurotrophins, derived from neurons, microglia, and astrocytes, that have been reported to regulate OL progenitor characteristics.** +, Positive regulation; −, negative regulation; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTFαR, CNTF-α receptor; CXCR, C-X-C chemokine receptor; EGF, epidermal growth factor; EGFR, EGF receptor; ErbB, ErbB tyrosin kinase receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; GDNF, glia-derived neurotrophic factor; GFRα1, GDNF family receptor α 1; IGF-1, insulin-like growth factor-1; IGF1R, IGF-1 receptor; NGF, nerve growth factor; NRG, neuregulin; NT, neurotrophic factor; OL, oligodendrocyte; PDGF-AA, platelet-derived growth factor AA; PDGFαR, platelet-derived growth factor-α receptor; SDF, stromal cell–derived factor; Trk, tyrosine kinase.

4.2. Astrocytes

Astrocytes are responsible for many different functions in the brain, including regulating the blood brain barrier (BBB), neurotransmitter uptake, synaptic transmission and the extracellular ion concentration [41]. It is now widely accepted that astrocytes also have direct influences on OL development and functioning [42]. Astrocytes and OLs are directly connected via gap junctions [43], formed by connexins (Cx) like the Cx26-Cx32 and the Cx30/Cx43-Cx47 channel [44]. Multiple studies showed that mutations in Cx47 and Cx32 result in myelin pathology [45,46]. The supportive role of astrocytes for OLs has also been illustrated in Alexander disease, in which dominant gain of function mutations in *GFAP* cause myelin defects [47]. Additionally, astrocytes regulate OL development via many secreted factors, including glycoprotein growth factors and neurotrophins (discussed below; **table 2**).

4.3. Microglia

Microglia have different roles in the CNS. The quiescent anti-inflammatory/immunoregulatory (M2) microglia remove damaged cells by phagocytosis following neuronal damage. However, the activated microglia, so-called pro-inflammatory (M1) microglia, display an inflammatory response after injury. Chronically activated microglia can cause damage to their surrounding cells via the release of potentially cytotoxic molecules, including pro-inflammatory cytokines, reactive oxygen intermediates, proteinases and complement proteins [48]. Microglia also regulate OL development, myelination and WM injury [49]. M2 microglia protect OL progenitors from apoptosis, and enhance OL maturation and myelination via the secretion of soluble factors [42,50] **(table 2)**, possibly by stimulating the synthesis of sulfatide [51]. Furthermore, they are involved in the promotion of myelination and axon regeneration **(table 2)**. During remyelination, microglia clear myelin debris [52], and their recruitment is regulated by astrocytes [53]. On the other hand, M1 microglia reduce IGF1 and CNTF production, secrete nitric oxide, and inhibit OL progenitor survival and OL differentiation [54-56]. Thus, microglia have both positive and negative effects on OLs, also depending on OL developmental stages [54].

5. Extracellular matrix

The ECM is a network of proteins and glycans that provides structural and biochemical support to surrounding cells [57]. The ECM is composed of factors that are secreted by surrounding cells, and consists of three compartments: the basement membrane, the perineuronal nets and the neural interstitial matrix. The basement membrane is positioned between the vasculature and the astrocyte endfeet, plays important roles in maintaining the BBB, and consists of molecular components like collagen, laminin, fibronectin and dystroglycan. The perineuronal nets surround neuronal cell structures, are responsible for synaptic stabilization, and consist of dense structures of proteoglycans. The neural interstitial matrix, on the other hand, is not strongly connected to the basement membrane or the perineuronal nets, has multiple ECM functions, and is a looser network of proteoglycans (e.g. tenascin), glycosaminoglycan (e.g. hyaluronan), proteins (e.g. collagen, elastin) and glycoproteins (e.g. laminin, fibronectin)

The ECM facilitates cell-to-cell communication and plays a role in the migration, differentiation and proliferation of neural cells during development [57]. Neural cells, including early OL progenitors and myelinating OLs, can bind to the ECM via cell-surface cellular adhesion molecules (CAM), such as integrins and cadherins, which are connected to intermediate filaments in their cytoskeleton [58-60]. Integrins have the ability to bind to proteins (e.g. collagen) and glycoproteins (e.g. fibronectin or laminin) in the ECM, and to integrins on other cells. Cadherins are calcium-dependent glycoproteins, which bind to the cytoskeleton through specific linking proteins called catenins, and tend to cluster at the intermediate cell junctions.

Because integrins and cadherins have an extracellular, a transmembrane and an intracellular domain, they have essential functions in mediating extracellular signals to intracellular signaling pathways [61]. Apart from the physical interaction, the ECM can also support in cell growth and survival by quick release of stored signaling molecules at specific time-points and in particular regions of the brain [62]. For example, protease activity can be triggered upon physiological changes without *de novo* synthesis, like matrix metalloproteinase 9 (MMP9) or MMP2, which in high amounts can cause basement membrane detachment and a compromised BBB.

Different proteins and glycoproteins in the ECM control OLs [63]. During development, N-cadherin is important for OL progenitor migration [64], and laminin stimulates postnatal oligodendrogenesis in the subventricular zone, as was shown in mice [65]. Alpha2-laminin-deficient mice show delayed OL maturation and accumulation of OL progenitors during the active myelination period [66]. Also in human, mutations in laminin genes lead to WM defects. Children with mutations in alpha2-laminin gene *LAMA2* show WM defects, next to defects in brain size [67]. The interactions between laminin and CAMs, like dystroglycan and integrin, may play important roles, as cleavage by metalloproteinase increases the number of OL progenitors [68], and administration of anti-sulfatide antibodies that modulates laminin-integrin interactions impedes OL differentiation and (re)myelination *in vitro* [69]. It is further shown that laminin acts on dystroglycan receptors to regulate processes of OL outgrowth and branching [70], as well as myelin production and stability [71]. Fibronectin is another glycoprotein that is well-studied for its function during OL development. It mediates proliferation of OL progenitors, but can form aggregates during inflammation-induced forms of demyelination, thereby impairing remyelination [72]. Furthermore, fibronectin can impair OL differentiation by inhibiting morphological changes in OLs that are required for the production of myelin sheets [73], possibly via mislocalization of MMP-9 activity [74]. The glycoprotein tenascin-C can inhibit processes of OL progenitor proliferation and maturation [75,76].

Another ECM molecule with clear regulating actions on OLs is the glycosaminoglycan hyaluronan. Hyaluronan is a large polysaccharide that forms complexes with proteoglycans in the ECM, and exists in a high (HMW) and in a low molecular weight form (LMW). Increased levels of hyaluronan in the neural interstitial matrix are described for WMDs, like Multiple Sclerosis (MS) and VWM. Patients with VWM, who show defects in OL progenitor maturation [18], have increased levels of HMW hyaluronan [77]. The HMW hyaluronan receptor CD44 regulates the cellular responses to hyaluronan, the assembly of hyaluronan-rich extracellular matrices, and mediates internalization of hyaluronan. Post-mortem brains of VWM patients also show increased levels of astrocytes expressing the CD44 receptor [77]. This indicates that astrocytes contribute to WM defects found in VWM patients, as was recently confirmed [19]. Also others have shown that increased HMW hyaluronan can inhibit OPC maturation *in vitro* [78]. Following CNS injury, HMW hyaluronan is rapidly degraded into LMW hyaluronan. Studies

in MS patients showed that the LMW hyaluronan can inhibit OL progenitor maturation via the toll-like receptor 2 (TLR2) [79].

In conclusion, since ECM molecules have important regulating functions during WM (re) generation, the composition of the ECM is an important determinant whether (re)myelination is successful. More ECM components that are known to influence OLs are listed in table 3.

Name	Binding partner	OL progenitor specification	Proliferation	Migration	Maturation	Survival	Myelination
chondroitin sulfate proteoglycan (CSPG)	protein tyrosine phosphatase sigma (PTPσ) receptor			-	-		-
hyaluronan (HA)	TLR2, CD44				-		-
laminin (Ln)	dystroglycan, integrins		+		+	+	+
osteopontin (OPN)	integrins		+			+	+
tenascinC (TN-C)	integrins		-	-	-	+	+
fibronectin (Fn)	integrins		+		-		+ / -
vitronectin (Vn)	integrins	+	+				+
matrix metallopro-teinases (MMPs)					+		+ / -
tissue inhibitor of metalloproteinase 1 (TIMP1)	CD63, integrins	+			+		+
polysialylated-neural cell adhesion molecule (PSA-NCAM)				+			-

Table 3 **An overview of the ECM components that have been reported to regulate OL progenitor characteristics.** This overview is based on 38 experimental studies, which showed regulating effects of the presented ECM factors on processes of OL development. This is not a complete list; many other studies have been performed. +, Positive regulation; –, negative regulation; ECM, extracellular matrix; OL, oligodendrocyte; TLR, Toll-like receptor.

6. Growth factors

6.1. Patterning factors

Many growth factors play an important role in the regulation of OL development from early to late stages. Studies in rodent and chick provided insight into the actions of different growth factors, their role at certain developmental stages, and their interactions via signaling pathways. During early development, several morphogens, also called patterning factors, are crucial for neural patterning and proper emergence of OL progenitor populations, such as sonic hedgehog (SHH), WNT, retinoic acid (RA) and bone morphogenic proteins (BMP) [80]. These specific sets of patterning factors generate OL progenitor populations with unique expression profiles. It is however unclear to what extent these initial identities are affecting OL functions later in life.

These patterning factors also show important regulatory functions at later stages of OL development. For example, while SHH is important in the specification and proliferation of early OL progenitors [81], it also promotes the differentiation towards OLs [82], and is a potent stimulator of remyelination [83]. On the other hand, BMPs can inhibit OL differentiation [84], work as a chemo-repellent in migration [85,86], and can impair remyelination [87]. And while WNT is a negative regulator of OL specification [88], its signaling is required for the timing of OL maturation and (re)myelination [89]. RA also has dual roles, as it can inhibit OL progenitor proliferation and differentiation [90], but has shown positive effect on remyelination [91].

6.2. Glycoprotein growth factors

Growth factors classically refer to the glycoprotein families, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF) and fibroblast growth factors (FGF). Binding of the growth factor to its receptor activates downstream signaling pathways, which often overlap or interact. Many of these growth factors act on OL progenitor proliferation, maturation, differentiation and myelin formation, and are secreted by astrocytes, microglia and / or neurons **(table 2)**.

Of the PDGF family, PDGF-AA is most known for its functions in OL development. PDGF-AA binds to the PDGFa receptor that is expressed by many OL progenitors [92]. Mice lacking PDGFa receptors demonstrate defects in OL progenitor proliferation and migration, and show precocious OL differentiation and myelination [93,94]. Furthermore, many FGF family members, which act via four FGF tyrosine kinase receptors (FGFR1-4), also have various functions during OL development, starting during early neural patterning. Of the FGF family, FGF2 is studied most and shows next to many other functions, important effects on OL proliferation, migration and myelination. Interestingly, mice lacking FGF2 lack a postnatal

peak period of oligodendrogenesis, but have a normal OL density in adulthood [95]. This indicates that, while FGF2 has important functions in OL development, other signaling cascades can compensate for lack of FGF signaling. Furthermore, similar to the FGFs, EGF has many effects on proliferation and differentiation of neural stem cells into OLs, both during embryonic development and in adulthood [96]. Additionally, IGF1 signaling can increase OL progenitor proliferation and maturation, as well as late-stage OL differentiation and myelination [97]. Overexpression of IGF1 can increase brain size and myelination of axons, and can result in an increased number of myelinated axons [98,99]. In conclusion, growth factors have many important actions on OLs that differ depending on the developmental stage.

6.3. Neurotrophins

Neurotrophins are a class of growth factors secreted by astrocytes, microglia and / or neurons, which can activate the p75 neurotrophin receptor (p75NTR) and the family of tyrosine kinase (Trk) receptors consisting of TrkA, TrkB and TrkC. The neurotrophins that have been shown to regulate OL development include BDNF, NT3, NGF and neuregulin. BDNF has clear effects on myelination, as it can increase expression of myelin proteins *in vitro*, and when it is absent reduced expression of myelin proteins and delayed myelination *in vivo* is observed [100]. NGF can bind trkA or p75NTR, and depending on downstream interactions with other signaling pathways, can either encourage survival or induce cell death of OLs [101]. Furthermore, since neuronal activity can regulate regional secretion of neurotrophins, these neurotrophins can also have crucial roles in deciding whether and when OLs myelinate certain axons [102].

7. Blood-derived factors

The blood transports many factors from the periphery to the brain, and from one brain region to another. The passage of the blood-derived factors into the brain edema is strictly regulated by the BBB, which is composed of endothelial cells, astrocyte end feet, pericytes, and the basement membrane. The cerebral endothelial cells have specialized tight junctions to limit the diffusion of toxic substances, and carry specific transporters with properties to selectively transport certain nutrients into the brain [103]. The astrocyte end feet interact with the endothelium via gap and adherens junctions, and help determine BBB function. The pericytes are however thought to be predominantly responsible for endothelial proliferation, vascular branching and the regulation of endothelial cell junction sites. Pericytes are flat contractile cells that develop around capillary walls, and are embedded in the basement membrane. The basement membrane provides the mechanical barrier that avoids the passage of macromolecules, and can support in functions such as cell migration.

One type of signaling molecule produced in the periphery with functions in the brain, is the thyroid hormone, such as triiodothyronine (T3). T3 is derived from the less active prohormone thyroxine (T4) that on its turn is produced by the follicular cells of the thyroid. The production of T3 and T4 is regulated by thyroid-stimulating hormone (TSH) secreted from the pituitary gland. Iodothyronine transporters regulate T3 and T4 transport over membranes. Upon binding of the nuclear thyroid hormone receptors TRα and TRβ, thyroid hormones regulate gene expression, including genes that are involved in OL development [104]. Several studies indicated that T3 is important in regulating the switch from OL progenitor proliferation to OL differentiation [105]. Furthermore, thyroid hormones promote the maturation of post-mitotic OLs by increasing the number of committed OL progenitors, and by upregulating the expression of various myelin genes [106,107]. Mice lacking the T3 receptor never switch from OL progenitor proliferation to OL progenitor maturation, and develop myelin defects [108]. Furthermore, it was shown that treatment with TRβ agonists increases differentiation of human OL progenitors *in vitro,* and can enhance myelination of the developing mouse brain *in vivo* [109]. In conclusion, thyroid hormones have important functions in stimulating, and especially timing of, OL differentiation **(table 1)**.

Apart from the physical barrier and transportation functions, the vasculature regulates local secretion of signaling molecules, such as chemokines by endothelial cells. While the presence of chemokines on vascular endothelium is extensively studied for its role in leukocyte extravasation, new studies also indicate a role for chemokines in OL development. Stromal cell-derived factor 1 (SDF-1) binds to chemokine receptor CXCR4, which is highly expressed on membranes of migrating OLs [110]. When Cxcr4 function in mice is lost, OL progenitors are not attracted to the vasculature and thereby lose migration behavior, as their physical support for movement is absent [110]. Also other chemokine receptors are expressed on developing OLs, like CCR3, which binds eotaxin chemokine C-C motif ligand 11 (CCL11). CCL11 readily crosses the BBB and has been shown to increase OL progenitor proliferation [111]. As more and more studies indicate a role for chemokines in OL development [112], it is expected that modulation of cytokine levels can contribute to a supportive microenviroment for myelin (re)generation.

8. Towards improved regenerative medicine

8.1. Identification of microenvironmental factors in WMDs

As many studies indicate that microenvironmental factors are important regulators of proper WM development, it is expected that in states of WM disease, changes in the microenvironment will determine successful or failed WM (re)generation. Indeed, all WMDs show small to large changes in the microenvironment.

One of the WMDs that is extensively studied for the involvement of microenvironmental factors is MS. MS shows high numbers of OL progenitors in active demyelinating lesions, but low OL progenitor numbers and complete demyelination in chronic-inactive lesions, implying that during the disease course the MS microenvironment is not successful in supporting OL maturation and myelination. Many immune cells, as well as astrocytes and microglial cells, infiltrate the lesions sites, which show active morphologies and changed expression profiles [50]. Active lesions are filled with pro-inflammatory M1 microglia opposing remyelination, while in chronic-active lesions, anti-inflammatory M2 microglia are present that secrete pro-regenerative factors. Chronic-active lesions in MS patients are further characterized by a changed ECM, such as increased levels of fibronectin and hyaluronan, in which activated astrocytes play an important role [50].

Another WMD that shows clear involvements of the microenvironment is VWM. As described above, recent studies confirm a major role for astrocytes in WM pathology of patients with VWM [19]. *In vitro*, OLs of VWM mouse models develop normally in the presence of healthy astrocytes, but healthy OLs show impaired maturation when co-cultured with astrocytes carrying VWM mutations [19]. This suggests that, in prospective cell therapies, grafts of healthy OLs would counter an aversive diseased microenvironment and therefore might fail successful myelination. Therefore, better understanding of the microenvironmental factors during normal OL development and WM formation, and how these interplay with intracellular signals, will be of utmost importance in understanding why WM (re)generation fails in WMDs.

8.2. Mouse modeling

There are different approaches to identify microenvironmental factors. Pathological studies on patient post mortem tissue have been very informative, but do not generate insight into the microenvironmental state during early disease states. Transgenic animal models that carry mutations in genes involved in functions of either intracellular OL or microenvironmental signaling, gave many new insights into OL and WM development [113]. There are animal models that representatively mimic the human disease and have been helpful in identifying new microenvironmental factors [19]. However, many disease mutations in rodent models do not

mimic typical characteristics of human pathology. For example, mouse models for PMD, like the jimpy mouse that has a spontaneous X-linked intronic deletion, or transgenic mice with increased copy numbers of *Plp1*, show impaired myelin formation [114,115]. However, *Plp* knockouts show almost no myelin defects, while *PLP* null mutations in humans result in PMD [116]. Secondly, a mouse model for Alexander disease overexpressing the human mutant *GFAP*, only resulted in Rosenthal fibers after 30% increase of the mutant protein, and did not resemble other pathological or clinical aspects of Alexander disease [117]. Another transgenic mouse model harboring human knock-in mutations of Alexander disease showed defects in neurogenesis and cognition, since the mutant GFAP is also expressed in neural stem cells [118]. However, although the mice developed Rosenthal fibers, the white matter architecture and myelination were unaffected, even when GFAP expression was increased to a lethal dose [119]. Furthermore, several mouse models for X-linked adrenoleukodystrophy (X-ALD), a lipid storage disorder that affects CNS myelin in humans, showed no neurological symptoms or myelin lesions in mice [120].

In conclusion, the species difference makes *in vivo* modeling of human WMDs in rodents still challenging. Furthermore, while in some cases experimental manipulation of the pathway led to an abnormal phenotype in mice, it is the question if this is the same pathological causality and mechanism causing disease in human patients. On the other hand, whereas modeling of genetic WMDs is often dependent on transgenic techniques in mice, models of WMDs that are caused by injury or inflammation can be accomplished in larger animals, e.g. pig or primates. As the brains of these animals are larger, have more convolutions, more WM, and a higher complexity, the results are also more translatable to humans.

8.3. iPSC-based modeling

Another strategy of dissecting microenvironmental factors involves *in vitro* modeling. Unfortunately, the isolation of human primary cells is restricted by the availability of tissue, and the variability between subjects is high. However, the use of human induced pluripotent stem cells (iPSCs) overcomes many of these issues. The invention of iPSC technology gave prospects for creating human *in vitro* models for WMDs. For example, iPSC-derived OL cultures created from PMD patients showed reduced numbers of OL progenitors, and abnormally branched mature OLs compared to controls [12,121]. Furthermore, iPSC-derived astrocytes from Alexander disease patients, with either the early onset or later onset variant of the disease, showed GFAP-positive aggregates, similar to (early stage) Rosenthal fibers present in the patient brain. Additionally, Alexander disease iPSC-derived astrocyte cultures suggested increased mTOR activation and upregulated cytokine secretion [122]. Also for X-ALD, iPSC-derived cultures showed typical human pathology [123]. In X-ALD patient iPSC-derived OLs, accumulation of the long fatty acid chains was observed, while X-ALD patient iPSC-

derived neurons were unaffected [123]. Furthermore, X-ALD iPSC models could discriminate between the more and less severe phenotypes [124].

While iPSC technology gives promising results, it is still challenged by high variability, and many iPSC models lack robust correlation between iPSC-derived OL defects and disease severity in patients. Also, current iPSC cultures often do not capture correct regional or functional subtypes of OLs, or do not model the different microenvironmental factors present in the diseased brain. Furthermore, enriched culturing conditions could mask disease phenotypes, and monocultures lack the interactions with their (pathological) environment.

8.4. Future of new stem cell technologies

While iPSC technology accelerated the development of new *in vitro* OL differentiation protocols [125-127], these differ in culturing methods, such as media compositions and growth additives during early neural induction up to late OL maturation. Many of the stem cell-derived OL populations are tested in myelination assays, including co-cultures with neurons, *ex-vivo* brain slices, and / or *in vivo* demyelination models [4,128,129]. However, as not all iPSC-derived OL populations are analyzed with the same assays and with standardized read-outs, it is unclear what the differences are between the populations. Besides, the brain shows OL heterogeneity [26,130,131]. Differences in OL proliferation and maturation, and the properties of myelin, including the myelin thickness, intermodal length and axonal thickness of myelinated axons, are all reported to differ between OL populations *in vivo* [23,132]. Furthermore, many WMDs show WM degeneration in particular patterns, suggesting that subpopulations of OLs are affected [133]. For that reason, we need specific iPSC-derived OL populations to study certain WMDs **(Figure 1)**. It is possible that the existing protocols generate stem cell-derived OL populations that mimic certain characteristics, or even specific OL subpopulations found *in vivo.* In order to model disease-specific OL (re)generation failure, and to produce OL populations which can replace regional-specific OLs, we need better understanding of how *in vitro* procedures influence OL identity and properties. Thus, standardized assays are required, including expression profiling of specific gene sets, and detailed analysis of the myelination properties **(Figure 1)**.

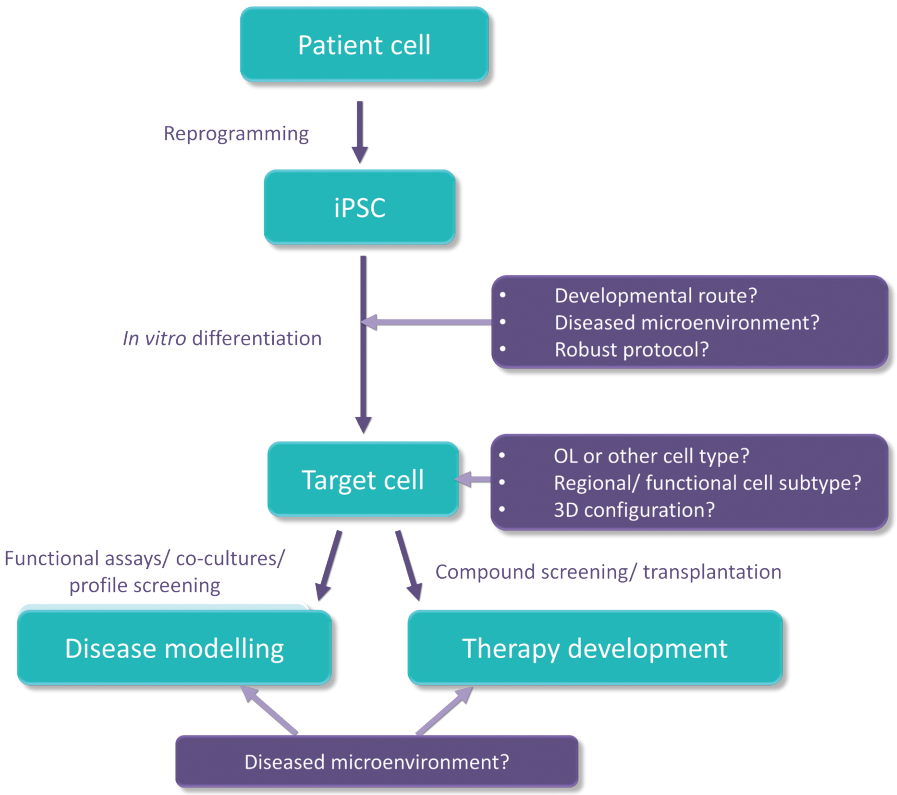

Figure 1. **Schematic overview of induced pluripotent stem cell (iPSC) technology in regenerative medicine and important considerations in studies of white matter disorders (WMDs) with microenvironmental involvements.** iPSC technology is broadly used to generate patient cells of interest in vitro, to study disease mechanisms using functional assays, co-culture systems, and profile screening, and to explore treatment options using compound screening and transplantation assays. However, existing protocols often do not take microenvironmental factors into consideration, which play important roles in WMD pathology. Furthermore, oligodendrocyte (OL) heterogeneity and non–OL-specific processes are involved. Therefore, in vitro stem cell protocols would benefit from the latest advances in the stem cell field, such as co-culture, three-dimensional (3D), and nanosubstrate techniques, toward the development of improved regenerative medicine for WMDs.

Many *in vitro* stem cell protocols focus on optimizing culture conditions to generate OL monocultures. These monocultures are not suitable for disease modeling or identifying interactive mechanisms between OLs and microenvironmental factors. Some disease phenotypes only become evident in complex or multicellular systems, and appear after failed interactions with the microenvironment. Furthermore, many protocols use multiple supplements to nurture the stem cell-derived OL products. It is possible that these well-

fortified conditions mask disease-associated phenotypes in patient-derived cultures. Therefore, in order to model such WMD phenotypes *in vitro*, we need dynamic culture systems that allow dissection of cell intrinsic from extrinsic aspects **(Figure 1)**.

As the importance of proper neuron-OL communication during myelination has already been appreciated for decades, there are many different neuron-OL co-cultures [134,135]. However, the evaluation of myelin formation in these cultures is still challenging for several reasons. At first, mixed cultures with random distribution of neural cell bodies and cellular processes are hard to control and analyze. Furthermore, it is challenging to manipulate cell-specific pathways, to quantify neuronal- *vs.* OL-related changes, and to uncouple direct from indirect (axonal-OL interactions) effects in traditional cultures. And lastly, most of these cultures contain one or more cellular products that are non-human. To our knowledge we still lack reproducible co-cultures of human OLs and human neurons. Additionally, many other microenvironmental factors determine OL functions, which should be included in these cultures. Therefore, we need human multicellular culture systems that are suitable to study WMDs, and that are robust and quantifiable **(Figure 1)**.

Artificial nanosubstrates helped the development of new tools to study myelination in complex cultures. For example, to study the effects of the biophysical properties of the axon on myelin formation, a neuron-free culture system was created. OLs were cultured in the presence of nanofibers, which substituted the axons and created a substrate for myelination [136]. Next to that OLs successfully myelinated nanofibers, it was shown that varying fiber diameters can regulate myelination processes [136]. Since no neurons were present in these cultures, other axonal/ neuronal signals were not responsible for the observed effects. Therefore, this *in vitro* system gave new methods to manipulate the biophysical properties of the axons, and can be used to study how administration of different factors affect myelination via direct OL signaling. Furthermore, this nanofiber-based culture is useful in modeling the regulating actions of ECM factors. It was shown that myelination of larger fiber diameters increased when nanofibers are coated with ECM glycoprotein laminin 2 [26,136]. In summary, while co-cultures give insight into complex intercellular signaling, fabrication of nanofibers can help to dissect OL intrinsic factors in presence or absence of different signals **(Figure 1)**.

Furthermore, the use of artificial nanosubstrates also gave new ways to compartmentalize different cell types and structures into separate chambers, while still allowing communication and interaction [137]. By separating neuronal cell bodies from axons, microfluidic chambers give the possibility to study OL-axon interactions without interference of neuronal cell bodies. It was shown that these myelinating co-cultures together with microfluidic technology are able to follow OL process extension and retraction, OL anchoring to axon, as well as myelin wrapping over long periods of time [138]. Thus, different artificial nanosubstrates can be used

to investigate the effects of microenvironmental factors in a controlled, time- and location-specific manner **(Figure 1)**.

Many of the latest technologies to improve stem cell cultures involve 3-dimensional (3D) conditions. 3D cultures more closely resemble the *in vivo* differentiation and microenvironment than the widely used flat 2D culture methods, by creating complex interactions between the neural cells [139]. One of the 3D culture approaches involves embedding of cells in a thick gel of different kinds of ECM proteins, e.g. Matrigel [140,141]. It was shown that 3D conditions encourage OL maturation [142]. The mechanical stiffness of the gel matrix is one of the properties that influences cellular processes. It was shown before that soft brain-like matrices (1.5 kPa) enhanced OL progenitor branching compared to more rigid substrates (30 kPa) [143]. Another 3D culture method involves free-floating aggregate structures, also called 'cerebral organoids' or 'spheroids'. In cerebral organoids the pluripotent stem cells differentiate into complex 3D structures with organization of different progenitor domains and brain regions [144]. Although current 3D cerebral organoids receive more attention for the modeling of early neural and neuronal structures, these 3D cultures also contain radial glial stem cells [145] and glial cells [146], and therefore have potential to study OL development. Interestingly, recent 3D models not only show presence of neuronal and glial cell types, but also the presence of endothelial cells [147]. Therefore, capillary-like networks in neural cultures containing developing OLs could aid the study of blood-derived factors on myelination. Since 3D cultures have complex interactions with the cellular and extracellular microenvironment that resemble important aspects of the *in vivo* situation, these are interesting tools in the study of microenvironmental factors. Next to 3D analysis *in vitro*, different studies show transplantation of stem cell-derived 3D products into animal models [148]. Monitoring of grafted patient-derived cells in a brain gives the possibility to study cellular processes in a living multifaceted microenvironment, and over longer time periods. Although standardization of 3D cultures is challenging, even more so than 2D cultures, these developments illustrate how 3D and complex culture techniques promise new tools to dissect and modulate microenvironmental factors in OL development **(Figure 1)**.

The design of good read-outs for complex multicellular cultures to study OL-microenvironmental interactions is also not straightforward. Upscaling to high throughput seems however possible with the introduction of multiwell-based platforms, including those for 3D culture [149]. As super-resolution microscopy has been evolving for years, the ability of screening complex cultures at high resolution, will make imaging of different OL-microenvironmental interactions possible. As myelin is necessary for the efficient and rapid transmission of action potentials, properties of neuronal activity are another way to analyze (re)generation of myelin. Integrated systems with multi-electrode arrays (MEA) and optical imaging tools are in development, and have interesting potential to study OL actions on neuronal network functionality. Furthermore, evolvements in RNA sequencing of single

cells has only started, and will aid in the identification of cell-specific pathways in complex systems. Overall, while the use of stem cell techniques in the study of WMDs increased enormously, recent developments of different technologies promise new tools to help us investigate the interactions between OLs and microenvironment in health and disease in more detail **(Figure 1)**.

9. Conclusions

It is known that the microenvironment, including connections with neighboring cells, factors provided by local cells, the ECM and blood-derived factors, is an important regulator of basic processes of OL development and function. The long list of known factors underscores the complex and dynamic interplay between the microenvironment and the intracellular state of OLs, which results in OL heterogeneity [26,130,131]. Also in the pathology of WMDs, the microenvironment is involved. To facilitate the development of new treatments for WMDs, it is of utmost importance to study how microenvironmental factors regulate normal and diseased WM development. Whereas future OL replacement therapies give great promises, these can only be successful if the host microenvironment is permissive for functional integration of the transplanted cells. New stem cell protocols and culture techniques can be helpful in dissecting and modulating microenvironmental factors, and to test new therapeutic targets, but need standardization to evaluate OL products for functionality and subtype specificity. To design better therapeutic strategies, we need to identify microenvironmental factors involved in failure of proper OL development and WM (re)generation in patients with WMDs.

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